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T Cell Vaccination in Multiple Sclerosis: A Preliminary Report

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Multiple sclerosis (MS) is a presumed autoimmune disease of the central nervous system. Inoculation of attenuated T cell clones recognizing immunodominant regions of myelin autoantigens can protect animals from the induction of experimental autoimmune diseases. In this phase one trial, we investigated whether inoculations with attenuated T cell clones are feasible in humans for eventual trials with autoreactive clones and whether there are any associated immunologic effects. A total of seven inoculations with attenuated, autologous T cell clones isolated from the cerebrospinal fluid in four subjects with progressive MS was performed. No untoward side effects were observed. Immunologic studies suggested that the inoculation of autologous activated T cell clones followed by partial, short-term, immunosuppression as evidenced by a decrease of subsequent responses to stimulation via the CD2 pathway and increases in the autologous mixed lymphocyte response. We conclude that the use of attenuated autoreactive T cell clones appears feasible for further clinical trials in humans with autoimmune diseases. © 1992 Academic Press, Inc.

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system with a presumed autoimmune etiology mediated by T cells (1). While nonspecific immunosuppression can alter the course of severe progressive MS, such therapies are associated with toxic side effects preventing their use in early disease (2, 3). A major goal in the treatment of autoimmune diseases is to develop antigen-specific, nontoxic forms of immunotherapy.

Inoculation of attenuated T cell clones recognizing the inciting autoantigen can specifically prevent the induction of experimental autoimmune disease models, including experimental autoimmune encephalomyelitis (EAE) (4, 5), adjuvant arthritis (6), and autoimmune thyroiditis (7). It has been shown that the mechanism for this protection involves both short-lived anti-activated T cell responses and long-lived anti-clonotypic T cell responses, thus the term T cell vaccination. Animal experimentation has indicated the safety of T cell vaccination using either fixed for irradiated T cell clones. It thus seemed justified to explore the potential of this experimental approach in subjects with severe, progressive MS.

Here we report the preliminary results of the first phase one investigation of T cell vaccination therapy in humans with the following objectives: (i) to examine the feasibility of inoculating T cell clones into humans and to determine how such clones should be selected, expanded, and attenuated; (ii) to study whether there is toxicity associated with inoculations into humans; and (iii) to define the immunologic responses to the inoculation of attenuated T cell clones using measures of immune activation.

METHODS

Selection of Patients

Four patients with severe chronic progressive multiple sclerosis participated in these phase one studies (Table 1). Patient selection was based on the diagnosis of definite multiple sclerosis with a decline of one point in the Kurtzke exended disability status scale (EDSS) in the previous year (2), the presence of white matter lesions on magnetic resonance imaging (MRI), and the failure of either steroid or cyclophosphamide treatments. Trials were approved by the Brigham and Women's Hospital Human Subjects Committee. Informed consent was obtained from each patient after explanation as to the highly experimental nature of the treatment. Patients Sh, Re, and Po had been previously treated with iv cyclophosphamide and prednisone while patient Mo was previously treated with oral prednisone. Patients had not received steroids for 3 months or cyclophosphamide for 1 year prior to the T cell vaccination. Five patients with severe chronic progressive multiple sclerosis not receiving any treatment were studied concurrently during the trial at weekly intervals as a control group for immunologic studies.

T Cell Cloning

Peripheral blood mononuclear cells (PBMC) and T cells were isolated from heparinized venous blood as previously reported (8). Cerebrospinal fluid (CSF) cells

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TABLE 1
Patient Clinical Data

Patient	Age	Sex	Disease duration	Kurtzke EDSS	Ambulation index
Sh	42	F	18 yrs	6.0	3
Po	45	F	25 yrs	7.0	8
Re	54	M	15 yrs	6.5	5
Mo	37	M	5 yrs	6.0	4

Note. Magnetic resonance scans revealed extensive areas of increased T2 signal intensity in the deep cerebral white matter which were predominantly periventricular in all four of the subjects.

were directly cloned, as previously described, at 0.3 cells per well with 10^5 autologous, irradiated (5000 rads) PBMC and phytohemagglutinin-protein PHA.P (1.0 μg/ml) (Wellcome, Dartford, UK) in 96-well V-bottom plates (0.1 ml/well) (8). Forty-eight hours later, 0.1 ml of media containing 10% IL-2 (delectinated column purified T cell growth factor, from ABI, Columbia, MD) and 50 units/ml of rIL-4 (Genzyme, Cambridge, MA) were added to each well. Cultures were fed with IL-2 and IL-4 every 3–5 days until ~Day 12, when all the wells were passed to 96-well U-bottom plates for microscopic analysis. Growth positive wells were scored macroscopically by an inverted microscope and transferred into V-bottom plates at 5000 cloned T cells per well and restimulated with 10^5 allogeneic, irradiated (5000 rads) mononuclear cells (from leukopacs) and PHA.P (1.0 μg/ml) in media containing IL-2 + IL-4. When there were 20–100 × 10^3 T cells per V-bottom well (usually 3–5 days), clones were transferred to flasks (no more than one-third full, standing) at a cell concentration of 0.5 × 10^6 cells/ml. T cell clones were restimulated every 10–14 days, as above, in V-bottom plates with 5000 cloned T cells per well, 10^5 allogeneic irradiated mononuclear cells, PHA.P, IL-2, and IL-4.

Selection of T Cell Clones for Vaccination

The study was begun at a time when immunodominant regions of individual myelin antigens (i.e., myelin basic protein) had not been defined. In the absence of known target antigens, clones were chosen on the basis of three characteristics. First was the expression of the CD4 determinant, which was based on animal experiments indicating that T cell clones efficacious for vaccination were all CD4 positive. The second criterion was that each T cell clone had good growth characteristics. This was necessary to permit expansion of the clone to a high enough number for use in these experiments. Further, recent observations in animals suggest that T cell clones with the best growth characteristics protected better in T cell vaccination experiments (4, 9). Lastly, clones were examined for the presence of common T cell receptor gene rearrangements indicative of a dominant T cell clonotype (oligoclonality) as previously described (8). This was done on the hypothesis that clonally expanded T cells in CSF or blood, which we have only observed in patients with MS and not other neurologic disease controls, are more likely to represent pathogenic T cells in the disease.

Preparation and Inoculation of Attenuated T Cell Clones

Five hundred thousand cells of each clone were restimulated with autologous feeder cells and PHA.P for 48 hr in 1 ml of standard media in polypropylene 15 × 100-mm-round bottom tubes (Falcon, Lincoln Park, NJ). Cells from five to seven clones for each subject were pooled to a total of 50 × 10^6 cells, centrifuged, then resuspended in 1 ml of 0.3% formaldehyde solution in phosphate-buffered saline (PBS) and incubated at 4°C for 15 min. Formaldehyde was chosen on the basis of previous investigations in animals (5). After incubation, clones were washed four times with PBS at 4°C and resuspended in 1.2 ml of PBS. An aliquot of each T cell preparation was tested for bacterial growth; cytomegalovirus, herpes simplex, adenovirus, and varicella/zoster isolation; and HTLV-1 virus by polymerase chain reaction as previously described (10).

Skin tests were performed with 50,000 T cell clones diluted in 0.1 ml PBS to test for immediate type hypersensitivity prior to each inoculation. A total of 4 × 10^7 T cell clones diluted in 1.0 ml of PBS were injected subcutaneously (0.5 ml injected per arm). This number of T cells was chosen by an extrapolation of vaccine doses effective in experimental animals on the basis of relative skin surface areas (m^2). The safer subcutaneous route of injection was chosen as experiments performed in rats showed that subcutaneous injections of attenuated T cell clones were as effective as intravenous injections (I. Cohen, unpublished). A total of seven inoculations were performed; subject Sh had three, Po had two, and subjects Re and Mo each had single inoculations. Patients were monitored for changes in granulocyte counts, lymphocyte counts, monocyte counts, hematocrits, platelets, prothrombin times, SGOT, SGPT, alkaline phosphatase, uric acid, BUN, creatinine, electrolytes, and titers of antibodies to hepatitis, HTLV-1, and HIV viruses at monthly intervals for 3 months then at half-year intervals for 1 year.

Measurement of Response to Vaccination

Phenotyping of PBL. Cytofluographic analysis of T cells was performed by means of direct immunofluorescence with fluorescein-conjugated anti- CD3, CD4, and CD8 mAb at a dilution of one-twentieth (Coulter Immunology), and dual staining for CD4 and CD45RA was performed using fluorescein-conjugated anti-CD4 and phycoerythrin-conjugated anti-2H4 mAbs. Back-

ground fluorescence reactivity was determined with phycoerythrin or fluorescein-conjugated mIg. Flow cytometric analysis was performed by using an Epics C flow cytometer (Coulter Electronics, Hialeah, FL).

Proliferative responses of PBL. Peripheral blood T cells were isolated prior to treatment and 3 days, 7 days, 14 days, 21 days, 1 month, and 3 months after the injection of attenuated T cell clones. T cells, at a final concentration of $5 \times 10^4/200$ μl in standard tissue culture media, were added to an equal number of irradiated autologous non-T cells and cultured in triplicate using 96-well U-bottom plates (Costar Corp., Cambridge, MA) with (i) anti-$T11_2$ + anti-$T11_3$ mAbs (1:100) (kindly provided by Dr. Stuart Schlossman, Dana Farber Cancer Institute, Boston, MA); (ii) anti-CD3 (kindly provided by Dr. Stuart Schlossman) (1:100) + PMA (1 ng/ml) (Sigma); or (iii) PHA.P (0.5 μg/ml) (Burroughs Welcome). The plates were incubated in 95% CO_2 and in a humidified atmosphere at 37°C for 4 days. Cloned T cells were tested for their ability to stimulate allogenic MLR. Wells were pulsed with 2 μCi [^{3}H]thymidine during the last 18 hr of culture prior to harvesting on an automated Titertek harvester. Tritiated thymidine uptake was measured in a LKB scintillation counter. Autologous mixed lymphocyte responses (AMLR) and responses to tetanus toxoid and mumps antigen were performed as previously reported for 7 days (11).

Responses to T cell vaccine. The ability of activated and resting T cell clones (stimulators) to induce the proliferation of autologous peripheral blood T cells was examined. T cells were considered to be in a resting stage 8–10 days following stimulation and activated cells were taken directly from the vaccination population both prior to and after attenuation with formaldehyde. Stimulator populations were frozen using standard procedures in 10% dimethyl sulfoxide and 90% fetal calf serum. All three stimulator populations included nonvaccine clones as controls. For the assay, 10^5 irradiated (5000 rads) stimulator T cells were incubated under conditions presented above with equal numbers of peripheral blood T cells for 4 days harvested at various times after the T cell inoculation. Incorporation of [^{3}H]thymidine was measured as above.

RESULTS

Our first objective was to examine the feasibility of T cell vaccination in humans. T cells expanded from the CSF by single cell cloning after PHA stimulation could be grown in sufficient numbers for inoculation into patients. After formaldehyde fixation for 15 min (attenuation), there was a decrease of the numbers of T cells in the preparation by approximately 50% and this number remained stable over the next 5 to 7 days prior to injection of the attenuated T cell clones. The formaldehyde fixation appears safe as no reactions were seen at the site of the inoculation. Since there was no obvious clinical benefit to multiple T cell vaccinations in the first two subjects, single inoculations were performed in subsequent patients.

The second critical objective of this study was to monitor for possible early side effects after the injection of attenuated cloned T cells. No immediate or delayed-type hypersensitivity reactions were observed on skin testing, even after two previous inoculations of attenuated T cells. Nor was any change observed in standard measures of systematic toxicity (listed under Methods). Monitoring for clinical signs of disease flare-up was performed at monthly intervals and with bimonthly MRI scans for approximately 6 months after each inoculation. There was no evidence for acute exacerbations of a disease activity associated with the inoculation. As this was an open trial, no conclusion as to treatment efficacy can be made.

The proliferative response to stimulation via the CD2 determinant, the T cell receptor/CD3 complex, or the mitogen PHA was measured after the first inoculation in three of the four subjects and after the second inoculation in one subject. In all subjects there was a >40% decrease in proliferation after stimulation via the CD2 complex 3 to 7 days after inoculation of attenuated T cells (Table 2). Although somewhat variable, consistent changes were not observed in six unvaccinated patients with chronic progressive multiple sclerosis whose anti-CD2 proliferative responses were measured weekly during the same time period, suggesting that the decreases observed in anti-CD2 responsiveness in vaccinated patients represented a response to treatment. T cell proliferation after TCR/CD3 stimulation with the phorbol ester PMA decreased in two subjects and increased in a third subject 7 days after the inoculation of attenuated T cells (data not shown). There were no consistent changes in either cell surface expression of CD4, CD8, or CD45RA determinants or in proliferation to the recall antigens tetanus toxoid or mumps virus (data not shown).

The proliferative response to self-MHC (AMLR) is decreased by approximately 50% in patients with chronic progressive MS and other autoimmune diseases (11–13) and has been thought to represent a dysregulation of the immune network (14). We investigated the AMLR in two subjects after the inoculation of attenuated T cells (Fig. 1). In both subjects the AMLR markedly increased from 7 to 14 days after inoculation and returned to baseline over the next 2 months; no such changes were observed in serial measurements of an unvaccinated patient. These and other immunologic changes were not associated with any clinical changes in disease activity.

In the EAE model, both specific (clonotypic) and non-

TABLE 2

Inhibition of Anti-CD2 Induced Proliferation after Inoculation with Attenuated Human T Cell Clones

Subject	Multiple sclerosis subjects treated with attenuated T cell clones (Δcpm) % decrease proliferation			
	Preinjection (Day 0)	Day 3	Day 7	Day 21
Mo	21,198	>0% (30,322)	43% (12,167)	>0% (28,328)
Re	49,871	62% (18,512)	17% (41,320)	>0% (55,989)
Po (first inoculation)	80,794	49% (40,847)	35% (52,895)	55% (36,184)
Po (second inoculation)	161,453	>0% (193,027)	32% (109,084)	45% (89,446)

	Multiple sclerosis subjects not treated with attenuated T cell clones (Δcpm) % decrease proliferation		
	Day 0	Day 7	Day 14
Control 1	105,771	30% (73,978)	>0% (127,857)
Control 2	92,586	12% (81,629)	2% (90,900)
Control 3	22,962	>0% (44,944)	>0% (44,100)
Control 4	83,761	25% (62,750)	>0% (67,552)
Control 5	44,554	>0% (45,551)	>0% (67,552)
Control 6	33,477	>0% (34,639)	>0% (49,391)

Note. Peripheral blood T cells were isolated either prior to or after injection of attenuated T cell clones on the days indicated. Data are presented in the same format of proliferative responses via the CD2 pathway using a combination of anti-$T11_2$ and anti-$T11_3$ mAbs (Δcpm = cpm in the presence of mAbs subtracted by cpm of identical cultures without mAbs) in both MS patients receiving the T cell vaccination and in six MS patients with a similar disease stage not receiving the inoculations measured weekly during the same time period of the investigation but on different days. Decreases of proliferation greater than 30% are in bold typeface. The standard errors of triplicate values for each time point were less than 10% and are not shown.

specific (ergotypic) proliferative responses are observed *in vivo* after vaccination with activated MBP-reactive clones (15, 16). In unvaccinated humans, nonspecific proliferative responses are observed *in vitro* in response to activated T cell clones (17). Thus a major immunologic question was whether there was specific recognition of the T cell clones used as vaccine in individual patients. This was investigated by measuring the recognition of irradiated resting and activated cloned T cells *in vitro* after T cell vaccination. The proliferative response to activated T cell clones was variable, although in some instances there appeared to be a decrease in T–T cell interactions 3 to 7 days after the inoculation of attenuated T cell clones, possibly related to the decrease noted in activation via the CD2 pathway (Fig. 2). By 14 days there was a variable increase in the proliferative response in two subjects to both T cell clones used in the inoculation and control T cell clones that were not used (Fig. 2). Additionally, there were no T cell anti-clonotypic responses observed in subjects after either two or three inoculations of the attenuated T cell clones. Circulating anti-T cell antibodies could not be detected, even with undiluted sera (data not shown). Thus, no T cell-specific response anti-clonotypic response could be demonstrated.

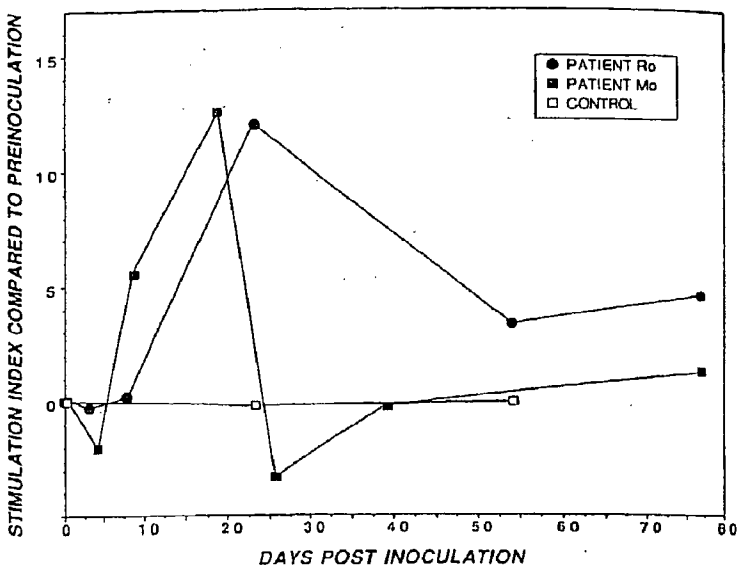

FIG. 1. Autologous mixed lymphocyte reactions were performed on two patients either prior to or after injection of attenuated T cell clones on the days indicated. A patient with chronic progressive multiple sclerosis not receiving treatment also had these analyses performed serially at the times indicated. Standard errors for triplicate wells were less then 15% and are not shown.

DISCUSSION

We present preliminary results of a phase one investigation of T cell vaccination therapy in patients with severe, chronic progressive MS refractory to other forms of immunosuppression. Autologous T cell clones could be isolated from CSF, expanded, attenuated, and inoculated subcutaneously without measurable short-term adverse effects. This study establishes the feasibility of this approach to immunotherapy.

Injection of attenuated autoantigen-specific T cells into naive animals induces an immune response that is capable of inhibiting the generation of new autoreactive T cells sharing the same antigen specificity (4–7). In EAE, this resistance appears to result from specific anti-clonotypic T cell response (15). In the present study, a transient increase in T cell responses was seen both with clones used for inoculation and with other autologous clones. Thus there was no evidence for a specific anti-clonotypic response as measured by proliferation.

We have previously reported that activated, irradiated T cell clones were strong stimulants to resting peripheral blood T cells. These T–T cell interactions are blocked by monoclonal antibodies recognizing adhesion molecules on the T cell surface, suggesting that CD2–LFA-3 and LFA-1–ICAM interactions are involved in this signaling mechanism (17). However, activated T cell clones also induce short-term immunosuppression in animal models of autoimmunity in a clonotypically independent fashion, which has been termed an "anti-ergotypic" response (16). Whether this response to activated T cells in animals parallels what

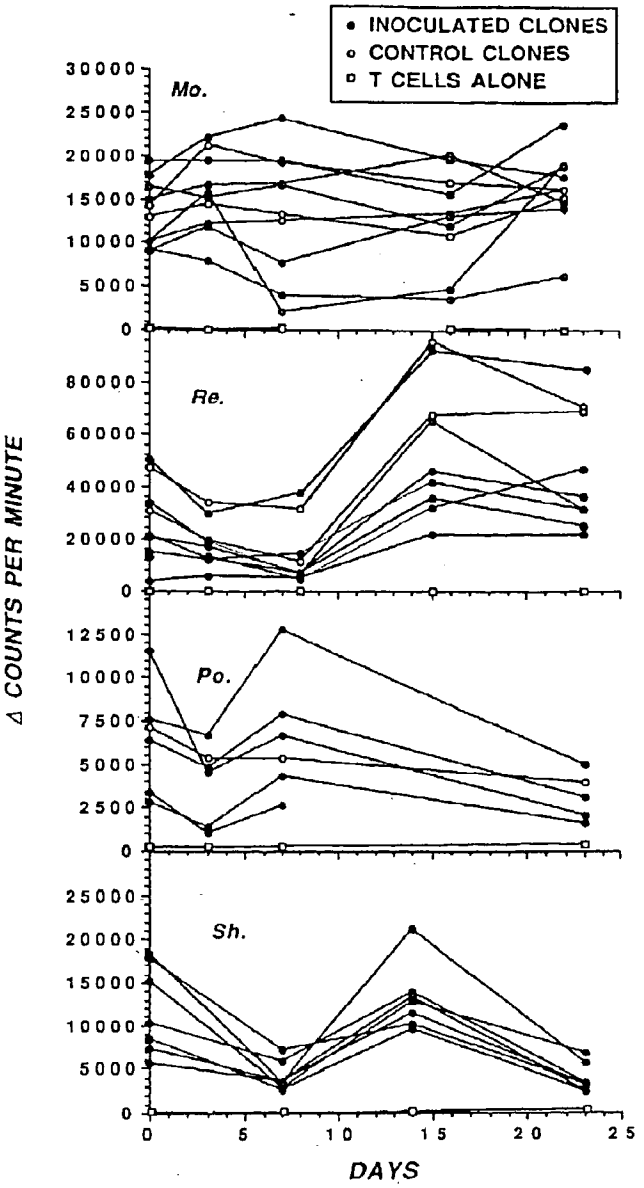

FIG. 2. The recognition *in vitro* of T cell clones inoculated *in vivo* was examined. For the assay, 10^6 irradiated (5000 rads) autologous activated T cells used in the inoculation (inoculated clones) or T cell clones derived from the cerebrospinal fluid at the same time but not used in the inoculation (control clones) were incubated with equal numbers of peripheral blood T cells at the times indicated after the T cell inoculation on Day 0. The first time point represents a preinoculation value. Wells were pulsed with 2 μCi [^{3}H]thymidine during the last 18 hr of culture prior to harvesting on Day 4. Standard errors for triplicate wells were less than 15% and are not shown.

we have observed in humans is not as yet known. After T cell vaccination, the responsiveness of circulating T cells to stimulation via the CD2 pathway and to a lesser extent the CD3 pathway was variably decreased. In contrast the AMLR, a measure of immune function that is decreased in autoimmune diseases, increased after T cell vaccination. These changes in immune function are compatible with the postulate that T cell vaccination can alter the immunoregulatory network for months despite a single inoculation. It can be further postulated that these nonspecific suppressor influences are normally generated *in vivo* to respond to T cell activation to downregulate the immune response. However, it must be cautioned that it is unknown what the precise relationship is between these measures of immune function and disease activity (1). Although unlikely, it should also be noted that despite the precautions used in preparing the attenuated T cells for vaccination, the immunologic effects we observed may have been related to the carry over of products in the media such as PHA. Double-blind clinical trials in the future should use media controls without T cells to address these issues.

There were no associated toxicities observed with the present investigation. Nevertheless, certain potential hazards may exist with T cell vaccination that should be addressed in future studies. First was the use in the present study of allogeneic feeder whole mononuclear cells necessary to allow the growth of T cell clones. If future studies are undertaken, leukophoresis with large numbers of autologous MNC feeder cells will be used. While in the present study T cell clones were screened by the polymerase chain reaction for the T cell tropic virus HTLV-1, in future investigations a larger panel of probes can be used to examine for evidence of viral contamination of T cell clones used for the inoculation.

It is important to note the highly experimental nature of these clinical trials. One rationale for performing these studies is provided by the fact that the present immunosuppressive treatments for severe progressive multiple sclerosis are associated with toxicities. The second rationale relates to the potentially disabling nature of progressive MS, making this autoimmune disease a more logical first disease to attempt T cell vaccination. The last rationale relates to the emerging concept that an understanding of the pathogenesis of autoimmune diseases will ultimately come from clinical experiments where specific immunotherapies can ameliorate disease progression. For these reasons, we felt justified in initiating these feasibility trials and strongly urge the coordination of future protocols among different laboratories for such trials.

The rationale for use of antigen nonspecific clones generated by single cell cloning relates to the previously described clonal dominance of certain T cells in the blood and CSF of a subgroup of patients with MS (8). Moreover, common T cell receptor (TCR) V_β usage among sets of oligoclonal T cells among different MS patients has been observed, although the antigen reactivity of these oligoclonal T cells clones is undefined (18). T cell vaccination was performed using these oligoclonal T cell clones described recently in more detail (18) when they were present, although whether such clones are relevant in the pathogenesis of MS is unknown.

Studies in EAE have demonstrated a restricted T cell response in terms of TCR usage to encephalitogenic

regions of the inciting autoantigen, myelin basic protein (19). More recently, immunodominant regions of myelin basic protein have been identified in humans by a number of investigators (20–22). As in EAE, the variable regions of the TCR β chain used to recognize immunodominant region of MBP appear to be somewhat limited, although not monoclonal (23). Additionally, in the EAE model, peptides derived from these variable region sequences can protect animals from active and passively induced disease (24, 25). Such studies suggest a logical choice of T cell clones and their cell surface molecules to be used in future investigations taking advantage of nonspecific suppressor properties of activated T cell clones and T cell reactivity to an autoantigen such as MBP where a natural occurring anticlonotypic response may be present. However, it should be cautioned that whether T cell-specific anticlonotypic responses occur in human T–T cell interactions and if in fact MBP is a target antigen in MS is not as yet known. In this regard, we have recently proposed criteria for the identification of the antigen in a T cell-mediated autoimmune disease, including an association between an immunodominant region of the presumed autoantigen with disease-associated class II MHC haplotypes; an increase in frequency of T cells with defined T cell receptors interacting with this immunodominant epitope; and inhibition of disease activity by inducing tolerance to the autoantigen (20, 26). As mentioned above the last criterion would indicate that *in vitro* experiments cannot provide convincing evidence for the association of an autoantigen with a disease but instead indicates the need for clinical trials in attempts to induce tolerance to an autoantigen or to target TCR that recognize a putative autoantigen. The use of attenuated autoreactive T cell clones or T cell vaccination provides such a potential mechanism.

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